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The Novel Marker, *DOG1*, Is Expressed Ubiquitously in Gastrointestinal Stromal Tumors Irrespective of *KIT* or *PDGFRA* Mutation Status

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We recently characterized gene expression patterns in gastrointestinal stromal tumors (GISTs) using cDNA microarrays, and found that the gene *FLJ10261* (*DOG1*, discovered on GIST-1), encoding a hypothetical protein, was specifically expressed in GISTs. The immunoreactivity of a rabbit antiserum to synthetic DOG1 peptides was assessed on two soft tissue tumor microarrays. The tissue microarrays included 587 soft tissue tumors, with 149 GISTs, including 127 GIST cases for which the *KIT* and *PDGFRA* mutation status was known. Immunoreactivity for DOG1 was found in 136 of 139 (97.8%) of scorable GISTs. All seven GIST cases with a *PDGFRA* mutation were DOG1-positive, while most of these failed to react for KIT. The immunohistochemical findings were confirmed with *in situ* hybridization probes for *DOG1*, *KIT*, and *PDGFRA*. Other neoplasms in the differential diagnosis of GIST, including desmoid fibromatosis (0 of 17) and Schwannoma (0 of 3), were immunonegative for DOG1. Only 4 of 438 non-GIST cases were immunoreactive for DOG1. DOG1, a protein of unknown function, is expressed strongly on the cell surface of GISTs and is rarely expressed in other soft tissue tumors. Reactivity for DOG1 may aid in the diagnosis of GISTs, including *PDGFRA* mutants that fail to express KIT antigen, and lead to appropriate treatment with imatinib mesylate, an inhibitor of the KIT tyrosine kinase. ***(Am J Pathol 2004, 165:107–113)***

Gastrointestinal stromal tumors occur in the wall of the bowel and have been proposed to arise from the interstitial cells of Cajal. The differential diagnosis of these tumors includes desmoid fibromatosis, Schwannoma, leiomyosarcoma, and, in some cases, high-grade sarcomas.[1] Accurate diagnosis of gastrointestinal stromal tumor (GIST) is important, because imatinib mesylate has been shown to significantly inhibit these tumors presumably through inhibition of the KIT tyrosine kinase receptor, which is highly expressed in these tumors.[2–5] As a result, the diagnosis of GIST relies heavily on KIT immunoreactivity. Current recommendations in the literature emphasize a diffuse, strong KIT immunoreactivity for the diagnosis of GIST.[6] CD34 immunostaining can also aid in the diagnosis, but a subset of cases is immunonegative while many other types of sarcomas are immunoreactive for this marker.[7–10] In the vast majority of GISTs, high levels of KIT expression are accompanied by a *KIT* gene mutation in exons 9, 11, 13, or 17.[11,12]

Recently, a subset of GISTs have been found to have *PDGFRA* mutations rather than *KIT* mutations.[13,14] Patients with GISTs containing mutations in *PDGFRA* may still benefit from imatinib therapy, but these tumors often fail to react with antibodies against KIT and hence may remain undiagnosed as GIST.[2] In addition, some GISTs with KIT mutations may have low KIT expression by immunohistochemistry yet will still respond to imatinib therapy.[15]

Although much work has been done on the biology of GISTs and KIT, additional insight has recently been gained through gene microarray studies.[16–18] These studies have identified a number of genes whose expression is relatively increased compared to other soft tissue tumors. This includes genes known to be involved with

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GISTs, such as *KIT* and *CD34*, but also includes a number of genes that have not been well characterized. We have generated an antiserum against one GIST-specific gene, encoding for the hypothetical protein FLJ10261, which we have named "Discovered on GIST 1" (DOG1). Using immunohistochemistry with this antiserum and *in situ* hybridization with *DOG1*-specific probes, we show that DOG1 is highly expressed not only in typical GISTs but also in *KIT*-mutation-negative GISTs.

Materials and Methods

Tissue Microarray (TMA)

The studies described here were performed with the approval of the Institutional Review Board at Stanford University Hospital. Two TMAs were used for this study. The first TMA contained 460 different soft tissue tumors from 421 patients, with each tumor represented by two cores. The samples were distributed over two array blocks that were constructed using a technique previously described[19] with a tissue arrayer from Beecher Instruments, Silver Spring, MD. Cores (0.6 mm) were taken from paraffin-embedded soft tissue tumors archived from the Stanford University Medical Center between 1995 and 2001. This array has also been used for characterization of apolipoprotein D expression.[20] The second TMA used GISTs that were obtained from the pathology archives of Oregon Health and Science University Hospital, the Portland VA Medical Center, and the Kaiser Permanente Northwest Regional Laboratory. This single-block array consisted of 0.6-mm cores from formalin-fixed, paraffin-embedded tumor assembled using a semiautomated tissue arrayer.[21] There was one core for each tumor, and all of the GISTs on this TMA were analyzed for mutations in exons 9, 11, 13, and 17 of the *KIT* gene using a combination of denaturing high pressure liquid chromatography and direct sequencing, as previously described.[13,22] *KIT* wild-type tumors included on the array were also screened for mutations in exons 12 and 18 of the *PDGFRA* gene.[13]

Antibody Generation

The cDNA-derived protein sequence of *DOG1* showed no significant homology with other genes, including the *KIT* gene. A rabbit polyclonal antibody was raised by injecting three peptides derived from the gene sequence (Applied Genomics Inc., Hunstville, AL). These peptides have no sequence homology to KIT. The peptides were synthesized by standard FMOC chemistry: peptide 1, EEAVKDHPRAEYEARVLEKSLK; peptide 2, DHEECVKRKQRYEVDYNLE; peptide 3, KEKVLMVELFMREEQDK. The peptides were conjugated to keyhole limpet hemocyanin and injected into two out-bred rabbits. The serum (S284) was harvested after the rabbits demonstrated a significant anti-peptide titer. Affinity-purified antibodies were obtained by passing the antiserum over an affinity column conjugated with the three peptides; bound antibodies were eluted with a pH gradient.

Immunohistochemistry

Primary antibodies were directed toward DOG1 (S284, rabbit polyclonal, 1:50; Applied Genomics Inc.) and KIT (rabbit polyclonal, 1:50; DAKO, Carpinteria, CA). Serial sections of 4 μm were cut from the tissue array blocks, deparaffinized in xylene, and hydrated in a graded series of alcohol. Staining was then performed using the EnVision+ anti-rabbit system (DAKO).

In Situ *Hybridization*

In situ hybridization of TMA sections was performed based on a protocol published previously.[23,24] Briefly, digoxigenin-labeled sense and anti-sense RNA probes are generated by polymerase chain reaction amplification of 400- to 600-bp products with the T7 promoter incorporated into the primers. *In vitro* transcription was performed with a digoxigenin RNA-labeling kit and T7 polymerase according to the manufacturer's protocol (Roche Diagnostics, Indianapolis, IN). Sections (5 μm thick) cut from the paraffin blocks, deparaffinized in xylene, were hydrated in graded concentrations of ethanol for 5 minutes each. Sections were then incubated with 1% hydrogen peroxide, followed by digestion in 10 μg/ml of proteinase K at 37°C for 30 minutes. Sections were hybridized overnight at 55°C with either sense or antisense riboprobes at 200 ng/ml dilution in mRNA hybridization buffer (DAKO). The following day, sections were washed in 2× standard saline citrate and incubated with 1:35 dilution of RNase A cocktail (Ambion, Austin, TX) in 2× standard saline citrate for 30 minutes at 37°C. Next, sections were stringently washed in 2× standard saline citrate/50% formamide twice, followed by one wash at 0.08× standard saline citrate at 50°C. Biotin-blocking reagents (DAKO) were applied to the section to block the endogenous biotin. For signal amplification, a horseradish peroxidase-conjugated rabbit anti-digoxigenin antibody (DAKO) was used to catalyze the deposition of biotinyl tyramide, followed by secondary streptavidin complex (GenPoint kit, DAKO). The final signal was developed with diaminobenzidine (GenPoint kit, DAKO), and the tissues were counterstained in hematoxylin for 15 seconds.

Scoring of Immunohistochemistry and in Situ *Hybridization*

Cores were scored as follows. A score of 0 was given for absent or insignificant staining: less than 5% tumor cells with light brown staining. A score of 1 was given for unscorable cores. A score of 2 was given for light brown stain in greater than 5% of tumor cells or dark brown stain in less than 50% of tumor cells. A score of 3 was given for dark brown staining in greater that 50% tumor cells. Nontumor cells and cells of unknown origin were not scored. The cores were independently reviewed by two pathologists (RBW and MvdR) and disagreements were reviewed together to achieve a consensus score.

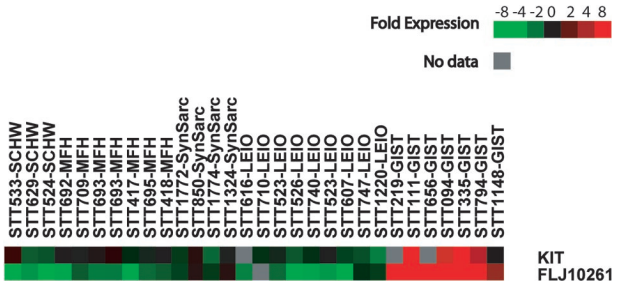

Figure 1. Gene array measurement of *KIT* and *DOG1* mRNA expression in 30 soft tissue tumors. Red indicates a relatively high level of expression whereas green denotes a low level of expression. Gene array data for STTs 524, 629, 417, 418, 219, 111, 656, 94, 335, 794, 1148, 850, 616, 710, 523, 526, 740, 607, and 1220 have been previously reported.[18]

Digital Image Collection and Data Analysis

To aid in the analysis of numerous tissue cores stained by immunohistochemistry and *in situ* hybridization, digital images were collected using the BLISS instrument (Bacuslabs, Lombard IL; *http://bacuslabs.com*). Scoring results were combined using Deconvoluter and represented in Treeview,[25] as shown on the accompanying website (*http://microarray-pubs.stanford.edu/tma_portal/dog1/*), where more than 4000 digital images are available.

Results

Previously, we examined the gene expression profile of GISTs using cDNA microarrays and identified a number of genes, in addition to the *KIT* gene, that demonstrated a specific pattern of elevated mRNA expression in GISTs.[18] Figure 1 shows the relative level of mRNA expression for one of these genes, *DOG1 (FLJ10261*), compared with *KIT* in a variety of soft tissue tumors, including those in the differential diagnosis of GIST. Searches failed to show any sequence similarity between the genes on either the DNA or protein level.

A rabbit antiserum was generated against synthetic peptides derived from the putative coding sequence of DOG1. Antiserum immunoreactivity was characterized on two separate TMAs containing soft tissue tumors. The first TMA contained 460 different soft tissue tumor samples representing more than 50 different diagnostic entities.[20] This array included 22 KIT-immunoreactive GISTs. The second TMA included 127 GIST cases for which the *KIT* and *PDGFRA* mutation status was previously determined. On this TMA there were 102 cases with an activating mutation in *KIT*, 8 cases with a mutation in *PDGFRA*, and 17 cases that were wild type for both kinases but nevertheless had clinical, histological, and immunophenotypic features typical for GIST.

In these two TMAs, 136 of 139 scorable GISTs (97.8%) demonstrated immunoreactivity with DOG1 antiserum (Figures 2 and 3, Table 1). The staining observed with DOG1 antisera appeared predominately localized to the plasma membrane (Figure 4A). In some very strongly immunoreactive samples, the subcellular distribution of the staining could not be evaluated (Figure 4B). Mast cells present in some of the samples, for example syno-

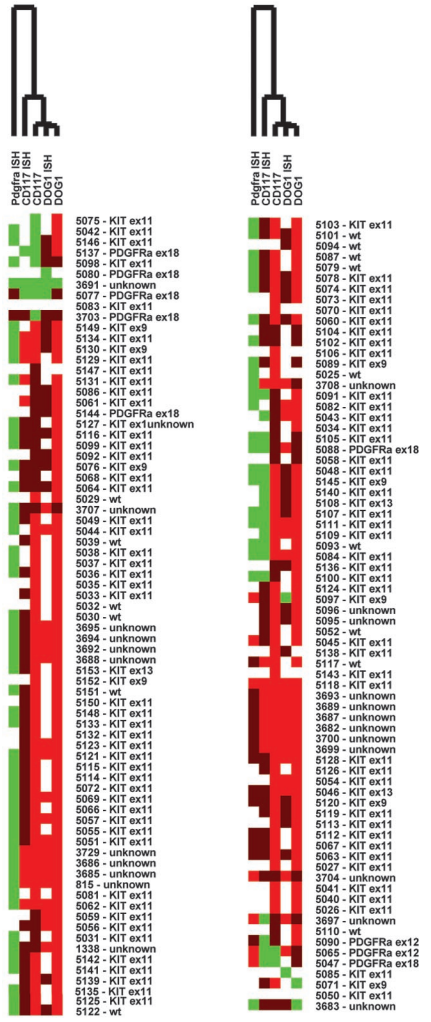

Figure 2. Hierarchical clustering of CD117 (KIT) immunohistochemistry, *CD117 in situ* hybridization, *PDGFRA in situ* hybridization, DOG1 immunohistochemistry, and *DOG1 in situ* hybridization. The results for GISTs on the two TMAs have been combined. Antisera or hybridization probes are in **columns**, tumors in **rows**. Bright red denotes strong reactivity, whereas dark red and green indicate low and absent reactivity, respectively. White means missing data.

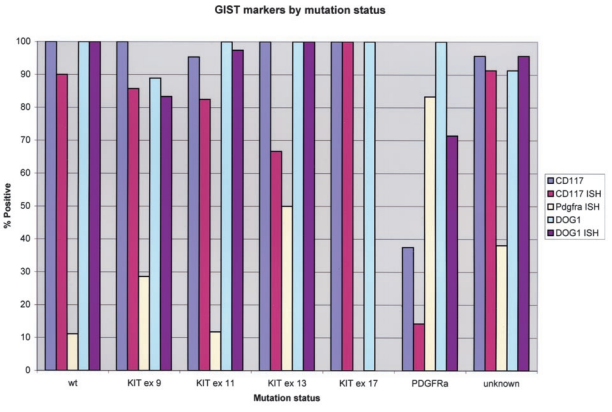

Figure 3. Staining results on GISTs for CD117 (KIT) immunohistochemistry, *CD117 in situ* hybridization, *PDGFRA in situ* hybridization, DOG1 immunohistochemistry, and *DOG1 in situ* hybridization in graphic form (see also Table 1).

Table 1. Staining Results for CD117 IHC, *CD117* ISH, *PDGFRA* ISH, DOG1 IHC, and *DOG1* ISH in Tabular Form (see also Figure 3)

Mutation status	CD117	*CD117* ISH	*PDGFRA* ISH	DOG1	*DOG1* ISH	
wt	14	10	9	14	3	Total scorables
	14	9	1	14	3	Total positive
	100	90	11	100	100	% positive
KIT ex 9	9	7	7	9	6	Total scorables
	9	6	2	8	5	Total positive
	100	86	29	89	83	% positive
KIT ex 11	86	57	51	81	39	Total scorables
	82	47	6	81	38	Total positive
	95	82	12	100	97	% positive
KIT ex 13	3	3	2	3	2	Total scorables
	3	2	1	3	2	Total positive
	100	67	50	100	100	% positive
KIT ex 17	1	1	1	1	0	Total scorables
	1	1	0	1	0	Total positive
	100	100	0	100	NA	% positive
PDGFRA	8	7	6	8	7	Total scorables
	3	1	5	8	5	Total positive
	37.5	14	83	100	71	% positive
Unknown	23	23	21	23	23	Total scorables
	22	21	8	21	22	Total positive
	96	91	38	91	96	% positive

IHC, immunohistochemistry; ISH, *in situ* hybridization.

vial sarcoma, were strongly immunoreactive as well (Figure 4C), whereas the same samples showed only weak staining in the mast cells with KIT antibodies. We confirmed these results with *in situ* hybridization studies (Figures 5 and 6). Interestingly, DOG1 antisera stained all eight scorable *PDGFRA*-mutant GISTs (one case from first TMA and seven cases from second TMA), whereas the KIT antibody staining was weak in three of these cases and negative in the remaining five. These findings were further extended by *in situ* hybridization with *PDGFRA* (Figure 6). *PDGFRA* expression was predominately, but not exclusively, present in the *PDGFRA*-mutant GISTs. Five of six (83%) scorable *PDGFRA*-mutant GISTs were positive for *PDGFRA in situ* hybridization (Figures 2 and 3, Table 1). In contrast, only 10 of 70 (14%) *KIT*-mutant and *KIT*-wild-type GISTs were positive for *PDGFRA in situ* hybridization. Correlation of *KIT in situ* hybridization with KIT immunohistochemistry was good, with the *in situ* hybridization signal detectable in almost all immunopositive cases (Figure 2). However, a difference was seen in the *PDGFRA*-mutant GISTs with regard to KIT expression. Three cases were immunopositive for KIT, but only one case was positive by *KIT in situ* hybridization. Hierarchical clustering analysis of immunohistochemistry and *in situ* hybridization data were performed as previously described.[25] Among these parameters—KIT immunohistochemistry, *KIT in situ* hybridization, DOG1 immunohistochemistry, *DOG1 in situ* hybridization, and *PDGFRA in situ* hybridization—the most distinguishing feature was *PDGFRA in situ* hybridization positivity (Figure 2), with overexpression of PDGFRA by *PDFGRA in situ*

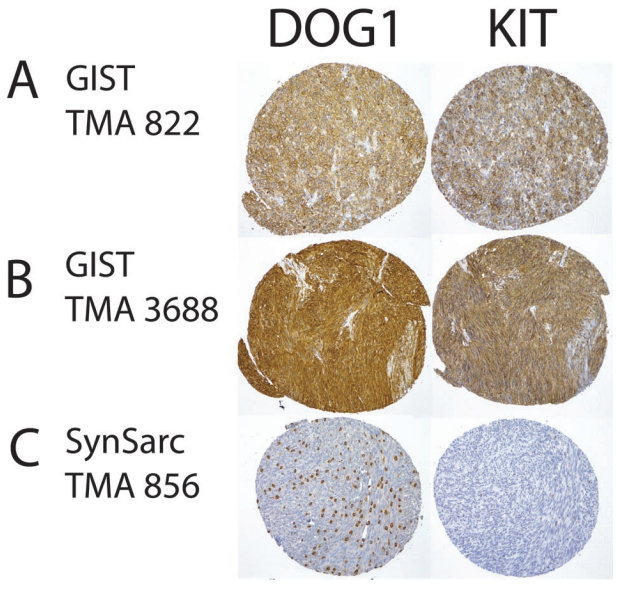

Figure 4. Immunohistochemical staining with anti-DOG1 serum (S284) and KIT on two GISTs [TMA 822 (**A**) and 3688 (**B**)] and a synovial sarcoma [TMA 856 (**C**)].

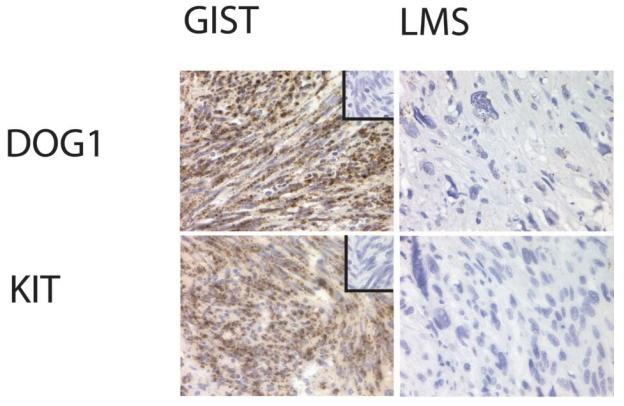

Figure 5. *In situ* hybridization of a GIST and leiomyosarcoma with antisense probes to *DOG1* and *KIT* on a GIST and a leiomyosarcoma (LMS). The corresponding negative control sense probes are included in the **inset** in the **top right** corner of the GIST sample.

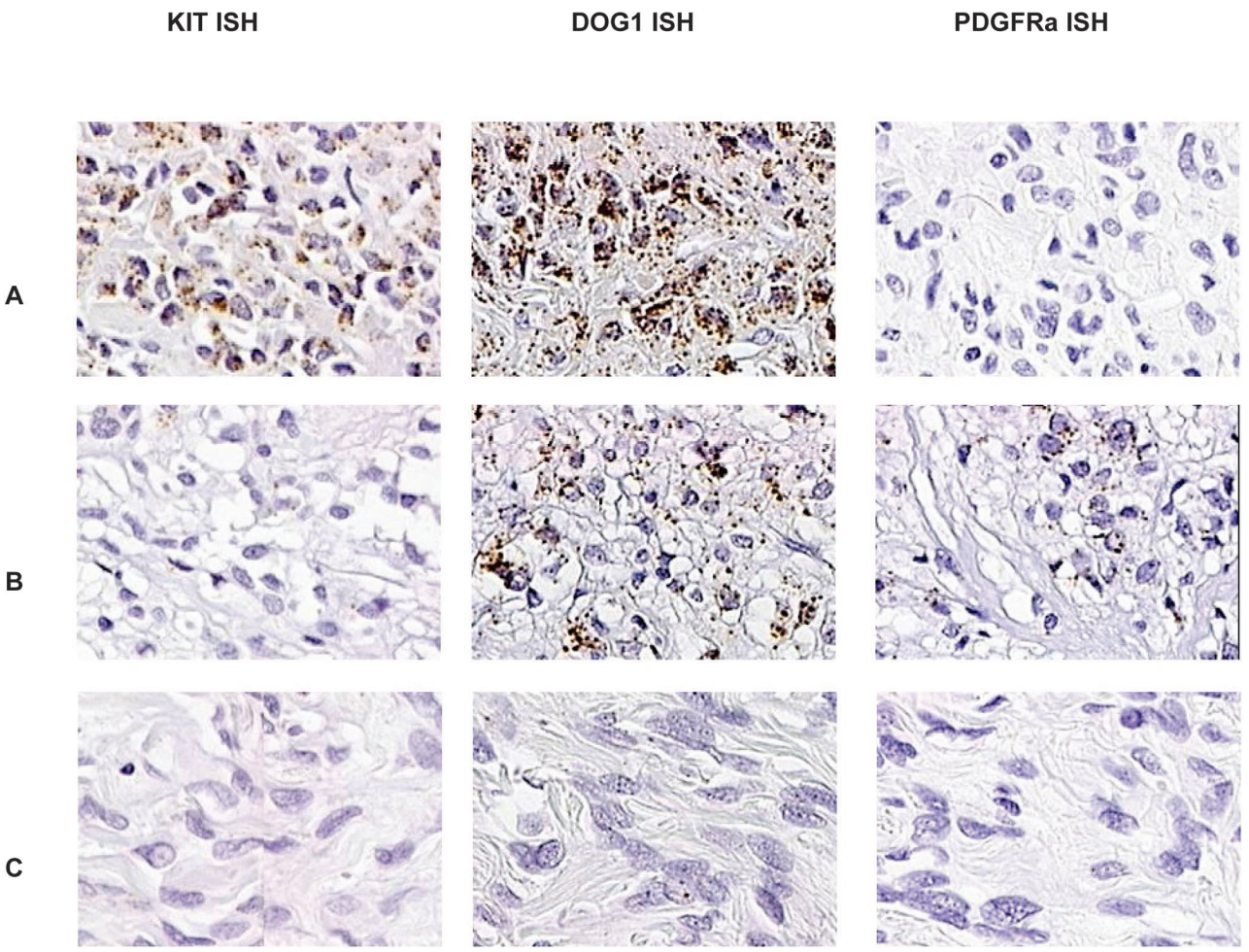

Figure 6. *In situ* hybridization of *KIT*, *DOG1*, and *PDGFRA* with GISTs. **A:** GIST with mutation in *KIT* shows positive *in situ* hybridization for *KIT* and *DOG1* but not *PDGFRA*. **B:** GIST with mutation in *PDGFRA* shows positive *in situ* hybridization for *DOG1* and *PDGFRA* but not for *KIT*. **C:** Negative control leiomyosarcoma.

hybridization seen in only in a small subset of GISTs. Images of all cores from both TMAs were digitally captured and are available at the accompanying website (*http://microarray-pubs.stanford.edu/tma_portal/dog1/*).

From the 460 tumor samples that were not classified as GIST in the first TMA, only four cases that were not histologically and immunophenotypically consistent with GIST were immunoreactive with DOG1 antiserum: one synovial sarcoma (1 of 20 = 5%), one (1 of 40 = 2.5%) leiomyosarcoma, one (1 of 4 = 25%) fibrosarcoma, and (1 of 9 = 11%) one Ewing's sarcoma/PNET. Of the 40 leiomyosarcomas, 17 originated in the abdomen and none of these were DOG1 immunoreactive. Other tumors in the GIST differential diagnosis failed to stain with the DOG1 antisera. These include desmoid fibromatosis (17 cases) and Schwannoma (3 cases). Parenthetically, under the staining conditions used, none of the fibromatosis cases were positive for KIT by immunohistochemistry or *in situ* hybridization. One leiomyosarcoma was positive for KIT immunohistochemistry only (TMA 3725). Interestingly, the staining was exclusively in a diffuse nuclear pattern. This tumor was negative for DOG1 by both immunohistochemistry and *in situ* hybridization and for *KIT in situ* hybridization.

Seven cases in the first TMA, not counted among the 22 unequivocal GISTs, showed histological features indeterminate between GIST and smooth muscle tumor. All of these tumors were located in the wall of the stomach or intestine, with four tumors from the stomach, one from the duodenum, one from the gastro-esophageal junction, and one from the rectum. All seven cases were negative for KIT by immunohistochemistry and thus might not be considered GISTs according to current recommendations.[6] However, four of the seven cases were positive by *KIT in situ* hybridization, while DOG1 immunoreactivity was seen in two cases, and all seven cases were positive for *DOG1* by *in situ* hybridization. Furthermore, two cases (TMAs 863 and 3696) were positive for *PDGFRA in situ* hybridization. Subsequent sequence analysis of cases 863 and 3696 revealed a point mutation and a deletion in exon 18 of *PDGFRA*, respectively. To date, such mutations have only been described in GISTs. We conclude that the seven KIT immunonegative cases with morphological features between GIST and smooth muscle tumor actually represent GISTs.

We also stained a TMA containing a spectrum of normal tissues with the DOG1 antiserum (data not shown). We observed staining in the epithelium of breast, pros-

tate, salivary gland, liver, stomach, testis, pancreas, and gallbladder. The pattern of DOG1 immunostaining of the interstitial cells of Cajal was similar to KIT. In addition, DOG1 antiserum reacted with a number of tumor cores in a carcinoma array, including some that did not stain with KIT antiserum (data not shown).

Discussion

GISTs have a high rate of local recurrence.[1] Imatinib, a small molecule inhibitor of several type III receptor tyrosine kinases, including KIT and PDGFRA, has demonstrated promise in controlling GIST growth.[3–5] The majority of GISTs (80 to 85%) harbor oncogenic mutations of KIT, and for this reason KIT has been regarded as the primary target for imatinib therapy. Indeed, initial trials of imatinib were limited to KIT-immunoreactive GISTs. Recently it was discovered that a subset of GISTs (5 to 7%) has activating mutations of PDGFRA.[13,14] Most of these tumors are weak or negative in immunostaining for KIT, which may lead to underdiagnosis and possible withholding of imatinib therapy. Furthermore, identification of *PDGFRA*-mutant GISTs requires molecular analysis, a laborious process that is not ideal for application in a routine clinical setting.

In this article, we demonstrate that a novel gene, *DOG1*, identified in a DNA microarray analysis of gene expression patterns as associated with GIST, is highly expressed in both *KIT*- and *PDGFRA*-mutant GISTs. Expression of DOG1 in GISTs was demonstrated both by immunodetection of the protein and by *in situ* hybridization. DOG1 immunoreactivity was assessed on two soft tissue tumor microarrays representing 587 soft tissue tumors, including 149 GISTs. Of scorable GISTs 97.8% demonstrated immunoreactivity with DOG1 antisera. Only four KIT-negative, non-GIST soft tissue tumors were DOG1 immunoreactive. Several GISTs with mutations in the PDGRFA gene were found to react only by *in situ* hybridization for DOG1 and to be negative for DOG1 by immunohistochemistry. Future studies are necessary to determine whether monoclonal antibodies against purified DOG1 might yield tools with sensitivity similar to that seen with *in situ* hybridization probes. We also confirm PDGFRA expression in a subset of GISTs using *in situ* hybridization. PDGRFA expression and KIT expression are not mutually exclusive. A subset of KIT-mutated GISTs expresses PDGRFA in addition to KIT while a subset of PDGRFA-mutated tumors also expresses KIT. These data were seen with both immunohistochemical and *in situ* hybridization techniques.

In addition to the marked similarity in reactivity for DOG1 protein on non-GIST sarcomas, DOG1 protein can also be seen in a subset of melanomas and germ cell tumors as has been described for KIT (West et al, in preparation). Furthermore just as seen with the KIT molecule, a variety of carcinomas also express DOG1. These tumors mostly overlap with the KIT-positive tumors. Although within the field of soft tissue tumors DOG1 expression seems quite specific for GIST, in a differential diagnostic setting DOG1 reactivity does not exclude carcinomas. Therefore additional markers such as keratin stains should be performed when the differential diagnosis includes carcinoma.

We also demonstrated the feasibility of assessing GIST markers by *in situ* hybridization on paraffin-embedded tissue. Correlation between immunohistochemistry and *in situ* hybridization for DOG1 on GISTs was excellent. In the case of KIT, the correlation was not as strong because of relatively weak or absent *in situ* hybridization signals in some CD117-positive GISTs. It is likely that this reflects lower sensitivity of the *KIT in situ* hybridization assay, although cross-reactivity of the CD117 antibody to another epitope on GISTs has not been excluded. *In situ* hybridization for *PDGFRA* proved to be valuable in identifying KIT-negative GISTs, although DOG1 immunohistochemistry was equally sensitive for these cases. Overall, we have found that *in situ* hybridization techniques are complementary to immunohistochemistry tests in the evaluation of GISTs.

DOG1 has been recently identified as a gene in the *CCND1-EMS1* locus on human chromosome 11q13, which is amplified in esophageal cancer, bladder tumors, and breast cancer.[26] Human DOG1 protein showed 89.8% total amino acid identity with mouse DOG1 protein, and also 58.4%, 38.3%, and 38.6% identity with human C12orf3, C11orf25, and FLJ34272/BAC03704 proteins, respectively. Sequence analysis predicts the presence of eight transmembrane-spanning segments. This correlates with our observations of the immunohistochemical localization to the cell membrane. *DOG1* may be part of an as yet unclassified ion transporter family.

Because the biological function is unknown, it is unclear why DOG1 is so widely expressed in GISTs. Two broad possibilities exist. It may be that the protein has a role in receptor kinase type III signal transduction pathways. On the other hand, DOG1 may be a fortuitous marker of the GIST phenotype, with no direct connection to the KIT and PDGFRA signaling pathways. The finding that mast cells are also immunoreactive for DOG1 tends to favor the former possibility.

In summary, we demonstrate that detection of a novel gene, *DOG1*, identifies the vast majority of both *KIT*- and *PDGFRA*-mutated GISTs. This may be of clinical value in identifying candidates for Gleevec therapy. As a cell membrane-associated protein, with markedly elevated expression in GISTs, DOG1 may also be a potential therapeutic target.

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